A TERNARY ACETYLCHOLINE–MANGANESE–ATP COMPLEX

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1. Introduction

Nucleotides have been implied on several occasions as being involved in the storage of acetylcholine in the nerve terminals [1–3]. Recently the association in solution of acetylcholine with ATP has been demonstrated by the shortening of the proton longitudinal relaxation times (T_1) of the acetylcholine protons produced by the addition of ATP [4]. However, in biological systems nucleotides usually exist as complexes with divalent metal ions, the formation constants for which are relatively high [5]. It is of interest, therefore, to examine whether acetylcholine can associate with a metal–nucleotide complex. Previous attempts to study this type of interaction by carbon-13 NMR have failed [6], probably because the diamagnetic alkaline-earth ions were used, and their effect on relaxation times is usually negligible. Paramagnetic ions, like manganese (II), e.g., greatly enhance the relaxation rates of nuclei in their vicinity and are often used as probes in studying interactions of biological interest [7,8]. Reported in this communication is an NMR study of the complex formation between acetylcholine and manganese–ATP.

2. Materials and methods

Chemicals were obtained from commercial sources. They were products of the highest available purity and were used with no further treatment. Solutions were made up in D_2O containing a small amount of *tert*-butanol, which served as an internal reference. Samples were prepared shortly before measurement and their pH was adjusted to 7.3 using NaOH.

Proton NMR spectra were recorded on a Varian T60 spectrometer operating at an ambient probe temperature of 39°C. The line-width at half-height was measured on a scale of 2 Hz/cm under conditions of slow sweep (0.2 Hz/sec). The radio frequency power level was kept well below saturation. The line-broadening was obtained from the measured line-width by subtracting the line-width in the absence of $MnCl_2$ and correcting for field inhomogeneity and bulk relaxation effects using the methyl signal of *tert*-butanol as a reference. For a typical titration the concentrations were 4.75 mM $MnCl_2$, 18.2 mM ATP disodium salt, and 20–110 mM acetylcholine chloride. Under these conditions the line-width of the *tert*-butanol signal is approx. 3 Hz compared to 0.7 Hz in the absence of paramagnetic ions. The line-broadening, Δ', is related to the increment in the transverse relaxation rate, $1/T_{2p}$, by $\Delta' = 1/\pi T_{2p}$. The experimental uncertainly in the Δ' values is estimated to be ± 0.2 Hz.

3. Results

As is well known [8] the transverse relaxation rate of a nucleus in a rapidly tumbling paramagnetic complex with negligible contact interaction is given by

$$1/T_{2M} = Dr^{-6}\tau_c \qquad (1)$$

where D is a constant of the system (in our case $D = 1 \times 10^{-30}$ cm^6/sec^2), r is the distance between the

nucleus and the paramagnetic center, and τ_c is the correlation time for molecular reorientation. Under conditions of fast chemical exchange between the complexed and uncomplexed species an increment in the relaxation rate is observed given by

$$1/T_{2p} = P_M/T_{2M} \quad (2)$$

where P_M is the fraction of complexed species. Thus in our case of acetylcholine (ACh) we have $P_M = [ACh]_b/[ACh]_t$, where the subscripts b and t refer to bound and total, respectively. It can be shown that if $[ACh]_t >> [ACh]_b$ and K_D is the dissociation constant for the complex between acetylcholine and Mn–ATP, $P_M = [M]_t/(K_D + [ACh]_t)$, where $[M]_t$ denotes the total concentration of the paramagnetic metal ion or, in our case, the total Mn–ATP concentration. By substituting in Eqn (2) and rearranging one obtains

$$T_{2p} = K_D T_{2M}/[M]_t + [ACh]_t T_{2M}/[M]_t \quad (3)$$

Thus a plot of T_{2p} versus $[ACh]_t$ at constant $[M]_t$ will be linear and from its slope and intercept the values of T_{2M} and K_D can be obtained.

Addition of ATP or of $MnCl_2$ taken alone to a solution of acetylcholine have no line-broadening effects. The Mn–ATP complex, however, produces a marked broadening of all signals with the N-methyls being broadened 3.65 times as much as the acetyl methyl. A representative spectrum is presented in fig.1. This effect clearly shows that there is a specific interaction between acetylcholine and the Mn–ATP complex. Upon the addition of an excess of $MgCl_2$ the signals narrow, demonstrating the displacement of the paramagnetic Mn^{2+} ion by the diamagnetic Mg^{2+} ion. This effect is also depicted in fig.1. The line-broadenings were studied at a constant Mn–ATP concentration (under our experimental conditions virtually all of the Mn^{2+} is present as the Mn–ATP complex [5]) and varying acetylcholine concentrations. The results are plotted in a semireciprocal form (cf. Eqn (3)) on fig.2. The results for both the acetyl and N-methyls were subjected to a simultaneous analysis so as to give the same dissociation constant according to Eqn (3). In this way the following parameters were obtained: $K_D = 220$ mM, T_{2M}(acetyl) = 3.33×10^{-3} sec, T_{2M}(N-methyl) = 0.91×10^{-3} sec. The straight lines drawn on fig.2 were calculated with these parameters. The standard deviations between the observed and calculated values are 8.0% and 4.5%, respectively, for the acetyl methyl and the N-methyls. Distances were calculated with Eqn (1), where a value of $\tau_c = 1.4 \times 10^{-10}$ sec was assumed for the ternary acetylcholine–Mn–ATP complex [9]. The results are r(acetyl) = 8.8 Å and r(N-methyls) = 7.1 Å. Because of the sixth root dependence of the distance upon the other

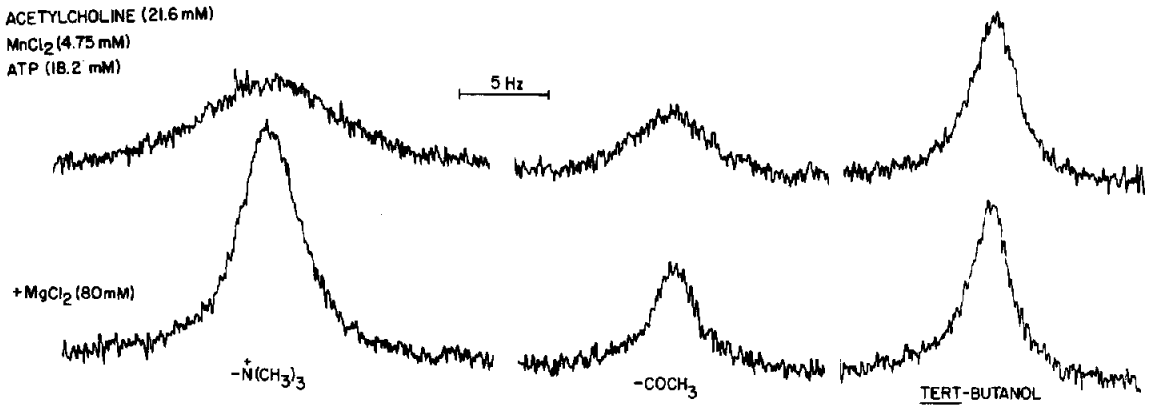

Fig.1. Proton NMR spectral lines of the acetylcholine (21.6 mM) N-methyls (left), the acetyl methyl (center), and the *tert*-butanol reference (right) in the presence of 4.75 mM Mn–ATP (top). The bottom spectrum shows the effect of addition of 80 mM $MgCl_2$ to the same solution.

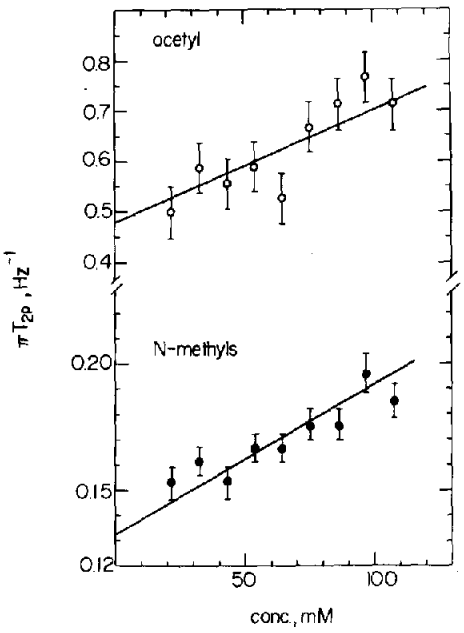

Fig.2. The reciprocal of the line-broadening, $1\ \Delta' = \pi T_{2p}$, as a function of acetylcholine concentration in solutions containing 4.75 mM Mn–ATP. The lines are calculated (see text).

parameters in Eqn (1) these values may be regarded as reliable particularly, the ratio of the distances, which is free of any assumptions.

4. Discussion

The formation of a ternary acetylcholine–manganese–ATP complex is clearly demonstrated by the present NMR results. The dissociation of acetylcholine from this complex is characterized by a relatively large constant (K_D = 220 mM), suggesting that the function of ternary complexes in the storage of acetylcholine may be marginal. On the other hand, if the binary acetylcholine–ATP complex is much stronger (and there are qualitative indications that this may indeed be the case [4], then the formation of a ternary complex with a divalent metal ion may play some role in the process of release of the neurotransmitter.

From the proton-manganese distances obtained in this study it is difficult to suggest a definite geometry for the ternary complex. Inspection of molecular models reveals that these distances are consistent with a model in which the manganese ion is associated with the α- and β-phosphates and the N-7 nitrogen of ATP [10,11], the positively charged *N*-trimethyl group of acetylcholine with the γ-phosphate, the acetyl group being hydrogen-bonded either to the ribose hydroxyls or to the adenine NH_2 group. It is emphasized that while such models make chemical sense they do not stem directly from the present results.

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